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CONTRACTING ORGANIZATION: V@ University of Ú^} } •^ |çæ ãæ
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REPORT DATE: T æ&@œFF

TYPE OF REPORT: Annual Û ~ { { æ^

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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1. REPORT DATE (DD-MM-YYYY)		2. REPORT TYPE		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
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1. Introduction

Targeted imaging agents use specific biomarkers that are present in tumor tissue to distinguish cancerous cells from their immediate benign environment. They are able to provide both structural and functional characteristics of the tumor such as shape, size, growth rate, and expression level of cell-surface markers. Today, the most commonly used x-ray contrast agents are iodine-based compounds [1]. However, the non-specific nature of these agents results in random vascular permeation, rapid renal clearance, and poor tumor-targeting potential.

The research aims to design an imaging platform using targeted gold (Au) nanoparticles (NP) to accurately determine the level of HER2 density in solid tumors. The nanoparticles will be synthesized in-house, and functionalized with an anti-HER2/neu affibody (for targeting) and a polyethylene glycol moiety (for stabilization). HER2/neu is a cell surface receptor protein that is overexpressed in roughly 25-30% of all breast cancers [2, 3]. The success of any therapeutic agent targeted to HER2 will depend greatly on the accurate assessment of the level of HER2 density in tumors. The long-term goal of this project is to develop a system that can identify those patients who will benefit the most from HER2-targeted therapies, and consequently reduce unnecessary side-effects, avoid false negatives, and ultimately improve patient survival rates.

2. Body

2.1 Research Overview

The initial research plans consisted of three major subsections:

- (i) Synthesize, functionalize and concentrate AuNP
- (ii) Characterize the structural and radiographic properties of the AuNP.
- (iii) Evaluate the *in vivo* effect of the nanoparticles: tumor-enhancement, biodistribution, and toxicity

In the period from Feb '09 to Feb '10, I had successfully managed to synthesize spherical AuNP using a modified Turkevich method. The AuNP were then surface stabilized (s-AuNP) using a heterobifunctional polyethylene glycol (PEG) chain, shown to enhance stealth characteristics and improve the circulation of nanoparticles *in vivo*. The physical characteristics (diameter, size distribution, zeta potential) of the AuNP agent had been determined through transmission electron microscopy (TEM), dynamic light scattering (DLS), and UV/Vis spectroscopy.

The majority of my effort this past year (for the period from Mar '10 to Mar '11) has been directed towards setting up the techniques and tools needed to complete the third major aim of the research proposal. I have also included a breakdown of the classes that I have taken to complete the didactic component of my PhD. Finally, I included an outline of the research I plan to pursue in the upcoming year.

2.2 Results

2.2.1 X-ray spectrum simulator

A simulation framework was developed in order to generate mammographic spectra that could be used to optimize the set of parameters needed to image the NP agent. This framework will help narrow down combinations of spectra, and filtration into a limited set that can be efficiently tested using physical phantoms. The framework was developed in MATLAB, with a graphical user interface (GUI) front-end to allow easy access of imaging parameters (see Figure 1). The GUI uses spectral parameters developed by Boone et al. [4] to create theoretical spectra of any peak voltage (kVp) that the user desires. The user can then select any combination of materials (filter, phantom) to be present in the beam path. The simulation software attenuates the input spectra by each of the materials and their corresponding thicknesses using:

$$I = I_0 e^{-\sum \left(\frac{\mu}{\rho} \right)_a \times \rho_a \times t_a}, \text{ (Eq. 1)}$$

where I is the spectrum after attenuation, I_0 : the spectrum before attenuation, $(\mu/\rho)_a$: the linear attenuation coefficient (in cm^2/g) of material a , ρ_a : density of material a (in g/cm^3),

and t_a : thickness of material a (in cm). The sum is performed over all the materials present in the beam.

Tube outputs (air kerma normalized by tube-current, mR/mAs) were measured for different target/filter combinations on both the GE Senographe 2000D and Hologic Dimensions systems. A liner fit was applied to model the relationship between the tube output and kVp for the various target/filter combinations in consideration (GE: Mo/Mo, Mo/Rh, Rh/Rh; Hologic: W/Rh W/Ag). This linear fit is then used in the simulation program to estimate the expected air kerma should be for a given mAs, and target/filter combination: K_{exp} . The ratio of K_{exp} to the air kerma calculated for the simulated spectrum (using the method of Boone et al. [4]) is then use to linearly scale the spectrum at each energy bin. This final spectrum has been calibrated to provide a sufficient simulation of the spectrum that would reach the detector should the input parameters be implemented in practice.

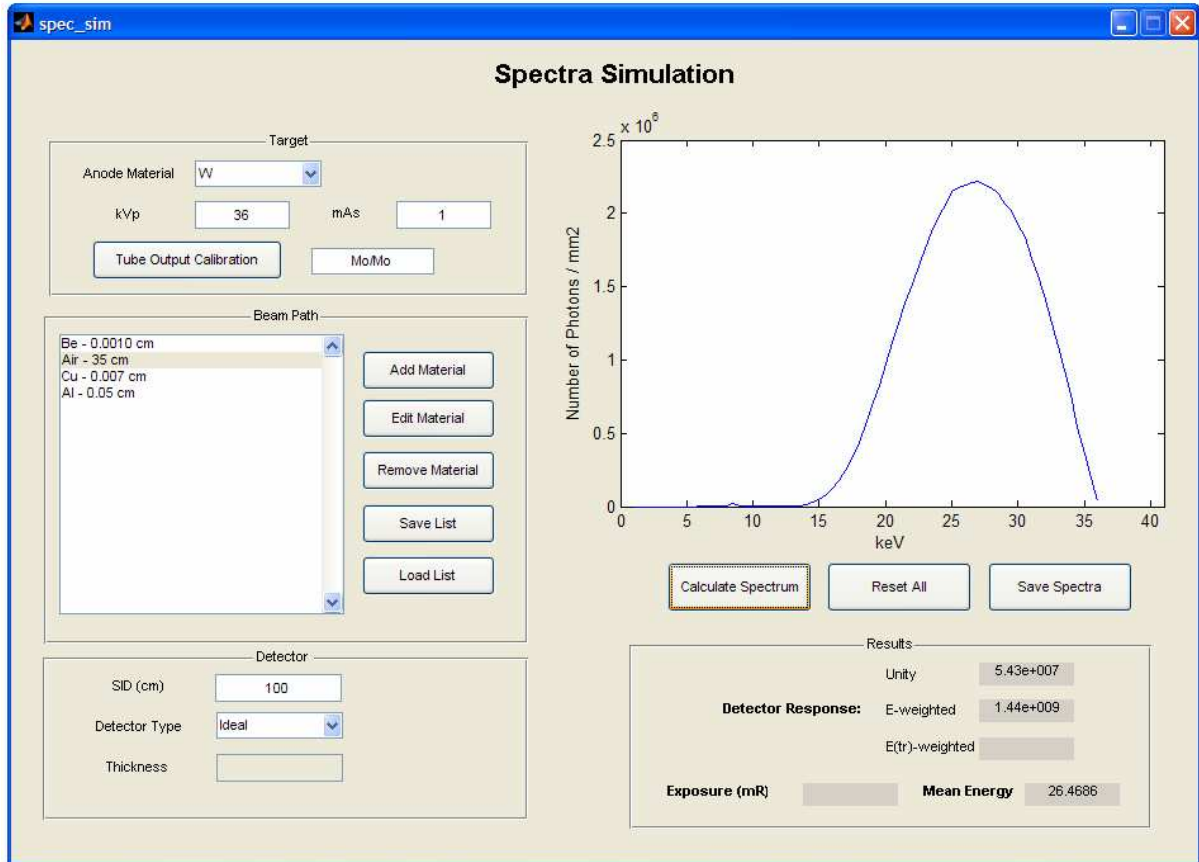


Figure 1. Simulation framework to generate mammographic spectra of different target , filter and bream path material combinations.

2.2.2 X-ray Spectrometry

A protocol was devised in order to quantify the concentration of AuNP solutions in terms of their equivalent iodine concentrations based upon the x-ray attenuation properties of the two materials.

The experimental design is shown in Figure 2. The spectral output of a laboratory x-ray system was measured using an Amptek XR-100T cadmium-zinc-telluride (CZT) x-ray spectrometer at a distance of 350 mm from the focal spot. The x-ray generator was from a Senographe 500T mammography unit. All exposures were performed with a Molybdenum target at 26 kVp and 50 mAs. Mo and Niobium (Nb) filters were used, each with a thickness of 25 μm . A simulation of the spectrum incident on the sample is shown in Figure 3. The k-edge of Nb (18.9 keV) is situated between the k-alpha and k-beta peaks of Mo. This results in an almost monoenergetic spectrum at the k-alpha energy peak of Mo (~ 17.4 keV).

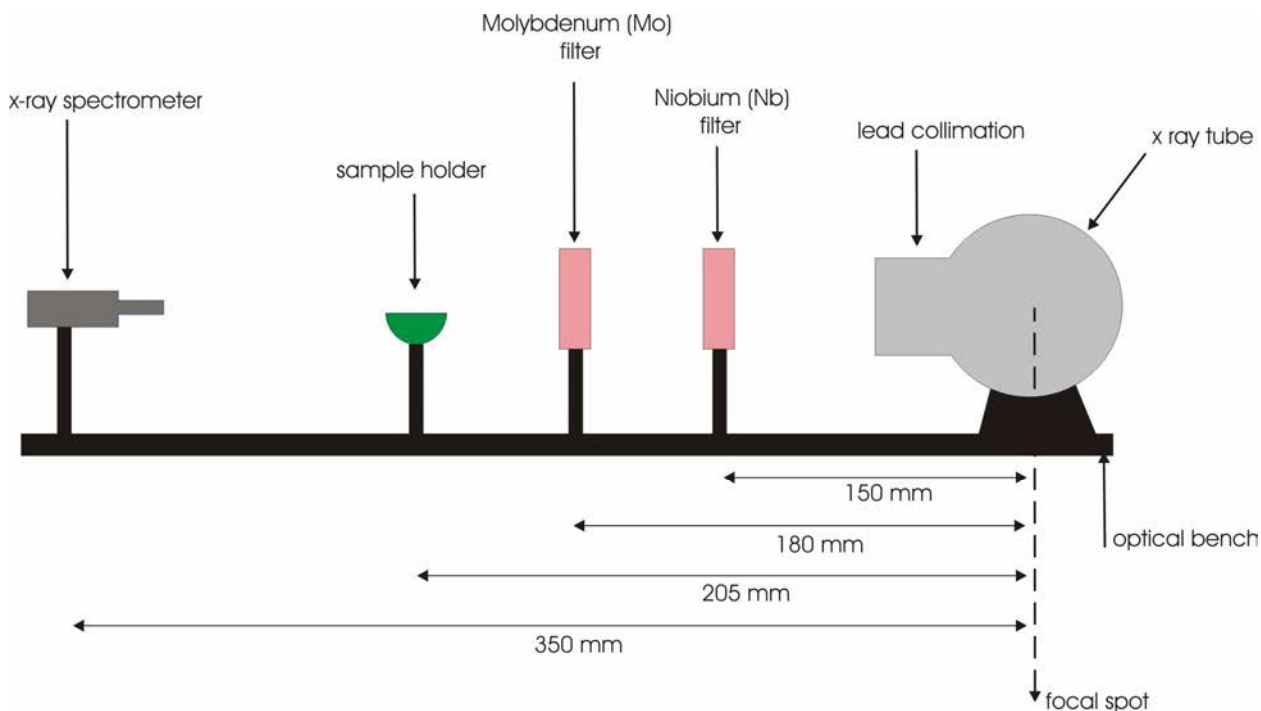


Figure 2. Experimental setup for x-ray spectrometry experiments.

A batch of Lugol's solution was synthesized to calibrate the spectrometer for various concentrations of iodine. The Lugol's solution was made by dissolving 1.08 g of potassium iodide in 8.5 mL of deionized water, to which 0.5232 g of solid iodine crystals were added. The concentration of iodine in the stock solution was calculated to be 158.74 mg I/mL. Dilutions of this stock solution enabled us to obtain solutions of iodine with concentrations ranging from 18 to 154 mg I/mL. 600 μL of each solution was pipeted into a microcentrifuge tube, and placed in the sample holder in the path of the primary beam. In addition, the experiment was run with tubes filled with water or air.

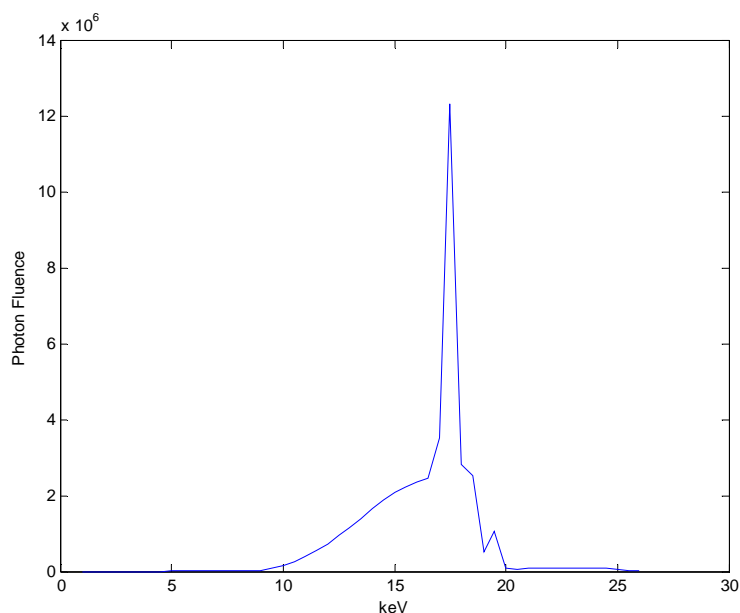


Figure 3. Simulation of the spectrum used in the spectrometry experiments. The parameters used were Mo/Mo (25 μ m)/Nb (25 μ m) at 26 kVp and 50 mAs.

Four separate spectra were obtained for each test solution (an example is seen in Figure 4). No corrections were applied to the spectra. The number of photons for each test sample (iodine solution, air, or water) was determined by summing the photon counts in each channel and then averaging this sum over the four exposure time points.

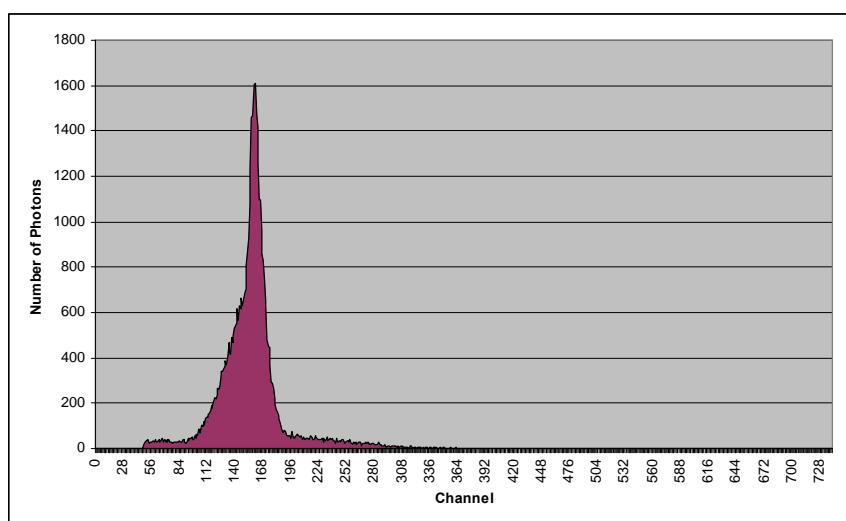


Figure 4. Experimentally-measured spectrum. Photon counts are obtained for each of 1024 channels. This is determined by the ADC gain set through the software.

The metric RU was defined as follows:

$$RU(i) = \frac{-\ln(Iodine) + \ln(Water)}{-\ln(Water) + \ln(Air)} \times 1000, \text{ (Eq. 2)}$$

Where *Iodine*, *Water*, and *Air* are the averaged number of photon counts for each sample. RU is graphed for the various concentrations of iodine in Figure 5. The relationship was modeled with a linear fit with an R^2 of 0.99 to be:

$$Iodine = \frac{RU + 119.56}{34.192}, \text{ (Eq. 3)}$$

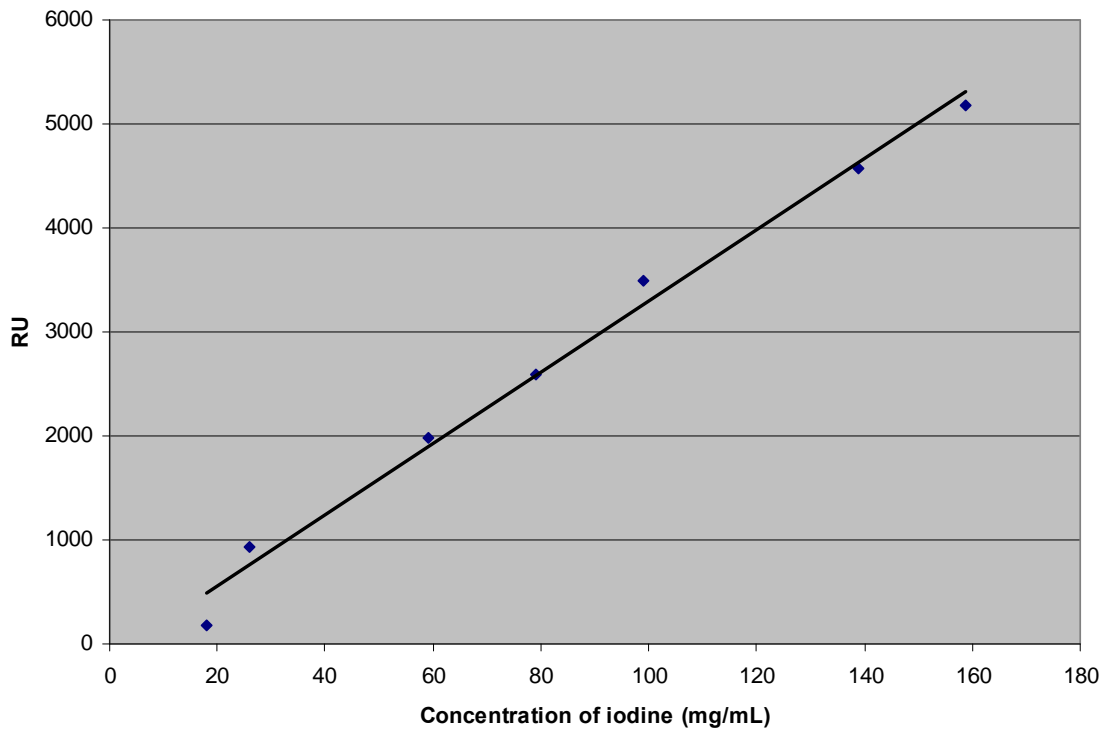


Figure 5. RU for various concentrations of Lugol's solution. The relationship was modeled with a linear fit.

Conclusions:

Equation 3 can be used to determine the equivalent iodine concentration of a sample of s-AuNP, given the RU for that solution of nanoparticles. This devised protocol thus allows for a quick and easy method to quantify the attenuation properties of the s-AuNP.

2.2.3 In vivo Imaging

Six to seven week old nude (nu/nu) mice were purchased from Charles River Laboratories and housed at the John Morgan Animal Housing Facility at the University

of Pennsylvania. The animals used in the following imaging studies were all weighed prior to injection and found to be between 23 and 26 g. The mice were anesthetized with 150 μ L of ketamine/acepromazine prior to all injections. All imaging was performed on Hologic Selenia Dimensions digital breast tomosynthesis unit at 25 kVp, 50 mAs and 35 μ m of rhodium filtration present in the beam. These parameters were determined beforehand by qualitatively inspecting projection images of anesthetized mice using the Hologic system.

Three series of injections and subsequent imaging were performed:

- Intraperitoneal (i.p.) injection with s-AuNP
- Intravenous (i.v.) injection with s-AuNP
- I.p. injection with Omnipaque (iodinated contrast agent, 320 mg I/mL)

I.p. injection with s-AuNP

The animal was injected via i.p. with 200 μ L of s-AuNP. The animal was then placed on the breast compression paddle of the imaging system with a geometric magnification of 2:1. A series of projection images was then obtained in 1-minute intervals over a time course of 30 minutes. Some representative images are shown in Figure 6.

Gold enhancement is seen immediately post-injection (b) in the i.p. injection image site. The NP agent was observed to slowly diffuse through the body cavity; however, no enhancement was observed in any of the major organs throughout the time course.

I.v. injection with s-AuNP

The animal was injected via i.v. with 200 μ L of s-AuNP and then placed on the breast compression paddle at a geometric magnification of 2:1. Projection images were then acquired in 1-minute intervals over a 20 minute period (Figure 7).

No enhancement was observed when the NP agent was injected i.v. into the animal. During the injection, the color of the animal's skin was observed to change rapidly to a dark-purple. The animal was observed daily up to 3 weeks after the experiment was completed. There were no signs of toxicity or adverse reaction to the NP agent, and the color of the animal eventually returned to normal two weeks post-injection (pink). . Our hypothesis is that the pH of the s-AuNP (~ 5.6) resulted in the localization of s-AuNP to the skin. This can hopefully be rectified with a more thorough membrane filtration of the s-AuNP.

I.p. injection with Omnipaque

The i.p. injection series was repeated with an iodinated contrast agent, Omnipaque 320 (320 mg I/mL). Iodine enhancement was observed immediately post-injection (Figure 8b) at the injection site, as with the s-AuNP agent. Significant enhancement was also observed in the bladder of the animal 26 minutes post-injection (Figure 8d). This may indicate that the iodinated agent is more rapidly cleared from the animal compared to the s-AuNP. However, extensive biodistribution studies would be needed to support this

theory. We attempted to repeat the i.v. injection series with the Omnipaque 320 but found that the iodinated agent was too viscous to be injected into the tail vein of the mouse.

Conclusions:

The s-AuNP shows potential for being used as an imaging agent in conjunction with digital breast x-ray imaging modalities. Similar contrast was observed to the clinically-implemented Omnipaque when the agents were administered into the body cavity of immunocompromised mice. This preliminary imaging study has helped me become accustomed to the techniques involved with *in vivo* imaging such as: animal handling, administration of anesthesia, imaging mice with a digital tomosynthesis unit.

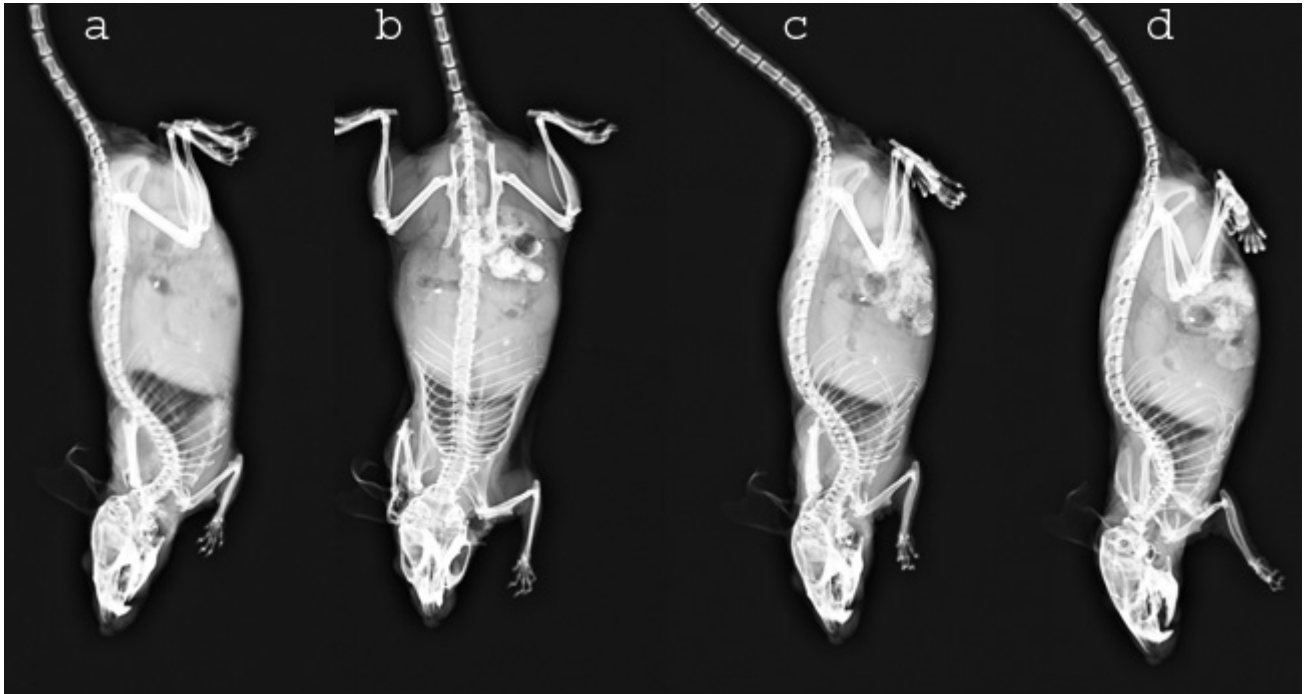


Figure 6. Intraperitoneal injection with s-AuNP: (a) Pre-injection, (b) 0 minutes, (c) 6 minutes and (d) 18 minutes post.

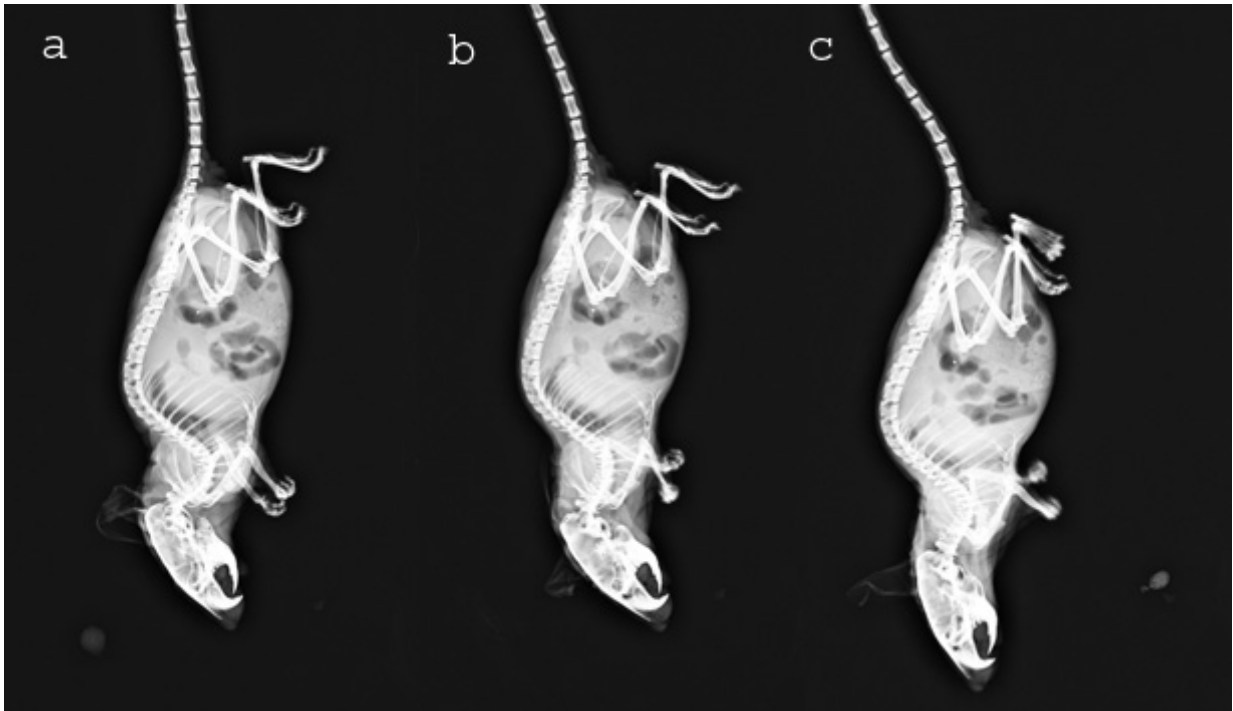


Figure 7. Intravenous injection with s-AuNP: (a) Pre-injection (b) 0 minutes (c) 19 minutes.



Figure 8. I.p. injection with Omnipaque 320. (a) Pre-injection, (b) 0 minutes, (c) 3 minutes, and (d) 26 minutes.

2.3 Didactic Component

I have taken several classes at the University of Pennsylvania in order to fulfill the course requirements for obtaining my Ph.D. in Bioengineering. I have compiled a list of some of the representative classes that I feel have helped me greatly in my research effort:

BE 545: Biomedical Image Analysis: This course dealt with the techniques that can be used in the analysis of medical images (the course dealt mainly with MR data). A lot of focus was placed on segmentation, registration and image transformation techniques.

BE 584: Math of Medical Imaging: The course dealt primarily with the mathematics behind image reconstruction of transmission x-ray tomography (computed tomography (CT); these principles are however easily transferable to mammography or breast tomosynthesis). Some of the topics covered include: Fourier and Radon transform, sampling theory, digital filtration and noise analysis.

BMB 585: Wistar Institute Cancer Biology Course: Signaling Pathways in Cancer: The course provided foundational information for information regarding the molecular basis of cancer. The main themes revolved around signal transduction pathways and mechanisms governing cell behavior in cancer. The course was structured so that each week, a guest lecturer from the department presented an ongoing topic of interest in the field of cancer biology as well as research they were conducting in their own labs. In this way, the class was a great example in how to teach the material but still keep it relevant to what is happening in the research field.

I have included a copy of my transcript which includes all the courses I have taken at the University of Pennsylvania in the Appendix A1.

I also successfully defended my Ph.D. thesis proposal titled “HER2/neu-targeted gold nanoparticles as molecular imaging agents for dual-energy computed tomography” on December 17th 2010. The proposal was approved by my thesis committee and as a result, my status has been upgraded to Ph.D. candidate.

2.4 Future Direction

The main focus of the next year will be to attach the targeting ligand (HER2 affibody) to the AuNP, and to test the resulting bioconjugated imaging agent in murine tumor xenografts. The HER2 targeting ligand will be purchased from Affibody Inc (Sweden) and will contain a terminal cysteine group. The thiol (-SH) group from the cysteine amino acid can be used to directly attach the affibody to the AuNP surface. The remaining surface area will be covered by a heterobifunctional PEG chain with a proximal thiol group for attachment to the gold surface and a distal infrared dye (HiLyte Fluor 647 – shown as purple dot in Figure 9) to aid with the *in vivo* biodistribution studies.

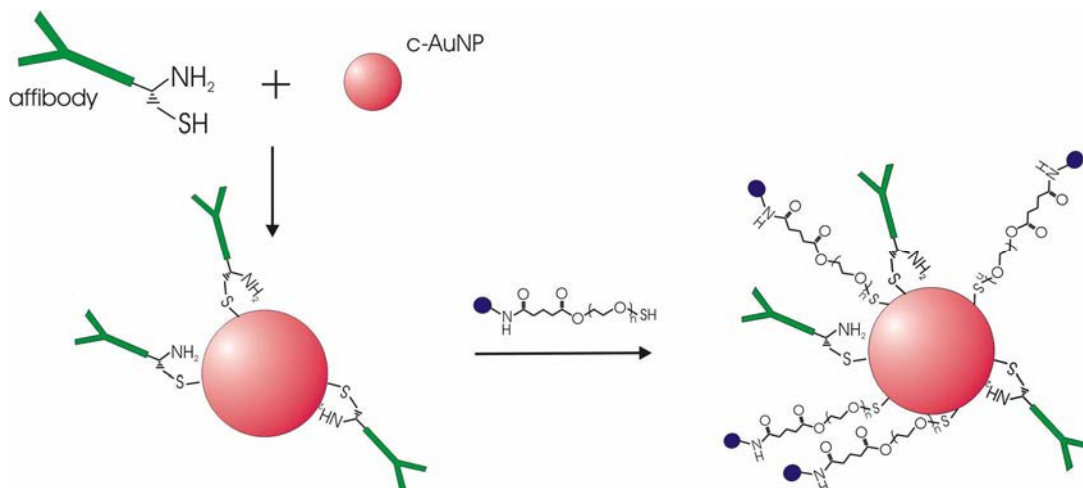


Figure 9. Functionalization scheme for attaching HER2 targeting ligand to AuNP.

The targeted AuNP agent will then be tested in murine tumor xenograft models to determine their ability to distinguish between HER2/neu- positive and –negative breast cancer cells.

3. Key Research Accomplishments

I have completed the necessary coursework for my Ph.D. and successfully defended my thesis proposal to my committee. I have developed a simulation tool that will allow me to optimize the imaging parameters needed for the AuNP imaging agent. I have also devised a protocol to estimate the iodine equivalent concentration of a AuNP solution using a spectrometer to determine the attenuated spectrum through a sample of the solution. This will help greatly when synthesizing new batches of AuNP, as a relatively easy way to compare concentrations between samples. I have also completed my first *in vivo* imaging study with a non-targeted s-AuNP. This preliminary study has taught me a great deal in regard to the skill set required as well as the considerations that have to be taken into account before an imaging regiment can be completed.

4. Reportable Outcomes

5. Conclusions

The work I have done over the past year has taught me a great deal in how I will proceed with my last year in this research grant. The imaging study I have performed will allow me to have an optimized protocol ready for when I will test the targeted AuNP agent *in vivo*. I have devised a protocol that will allow me to quantify the concentration of a batch of AuNP solution in terms of its equivalent iodine concentration. Finally, when fully matured, the x-ray spectrum simulation software will provide a powerful tool to be able to simulate x-ray transmission image acquisition techniques (CT, DM, and DBT).

The long term goal of this research project is to develop HER2-targeted AuNP agents will be able to quantify the expression levels of HER2 in primary breast tumors *in vivo*. Such an imaging agent would be able to differentiate those patients that would benefit most from HER2 therapy from those who wouldn't and thus provide personalized cancer therapeutics.

6. References

1. Rode, U. and R. Muller, *Transformation of the ionic X-ray contrast agent diatrizoate and related triiodinated benzoates by Trametes versicolor*. Appl Environ Microbiol, 1998. **64**(8): p. 3114-7.
2. Baselga, J., A.D. Seidman, P.P. Rosen, et al., *HER2 overexpression and paclitaxel sensitivity in breast cancer: therapeutic implications*. Oncology (Williston Park), 1997. **11**(3 Suppl 2): p. 43-8.
3. Baselga, J., D. Tripathy, J. Mendelsohn, et al., *Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer.[see comment]*. Journal of Clinical Oncology, 1996. **14**(3): p. 737-44.
4. Boone, J.M. and J.A. Seibert, *An accurate method for computer-generating tungsten anode x-ray spectra from 30 to 140 kV*. Med Phys, 1997. **24**(11): p. 1661-70.

7. Appendix

7.1 A1

Transcript from the University of Pennsylvania for courses completed during my Ph.D.

***** DEGREES AWARDED *****

05-19-08 MASTER OF SCIENCE IN ENGINEERING

***** UNIVERSITY OF PENNSYLVANIA COURSE WORK *****

Fall 2006		ENGINEERING & APPLIED SCIENCE MASTERS				
BE	580	MEDICAL RADIATION ENG	1.00	CU	A-	
CAMB	617	EMERGING INFECTIOUS DISE: Emerging Infectious Diseases	1.00	CU	B-	
ENM	510	FNDATIONS OF ENG MATH I	1.00	CU	A	
		Term Statistics:	3.00	CU	GPA 3.47	
		Cumulative:	3.00	CU	GPA 3.47	
Spring 2007		ENGINEERING & APPLIED SCIENCE MASTERS				
BE	899	INDEPENDENT STUDY	1.00	CU	A	
BIOL	540	GENETIC ANALYSIS	1.00	CU	B+	
ESE	511	MOD OPT&IMAGE UNDERSTND	1.00	CU	A	
		Term Statistics:	3.00	CU	GPA 3.77	
		Cumulative:	6.00	CU	GPA 3.62	
Fall 2007		ENGINEERING & APPLIED SCIENCE MASTERS				
BE	899	INDEPENDENT STUDY: Imaging Acquisition	1.00	CU	A	
BE	899	INDEPENDENT STUDY	1.00	CU	A	
BE	999	THESIS/DISSERTATION RSCH	1.00	CU	S	
		Term Statistics:	3.00	CU	GPA 4.00	
		Cumulative:	9.00	CU	GPA 3.71	
Spring 2008		ENGINEERING & APPLIED SCIENCE MASTERS				
BE	545	BIOMED IMAGE ANALYSIS	1.00	CU	A	
BE	999	THESIS/DISSERTATION RSCH	1.00	CU	A+	
		Term Statistics:	2.00	CU	GPA 4.00	
		Cumulative:	11.00	CU	GPA 3.77	
Fall 2008		ENGINEERING & APPLIED SCIENCE PHD				
BE	584	MATH OF MED IMAGE/MEASUR	1.00	CU	B	
BE	699	BIOENGINEERING SEMINAR	0.50	CU	S	
BE	999	THESIS/DISSERTATION RSCH	3.00	CU	A+	
		Term Statistics:	4.50	CU	GPA 3.75	
		Cumulative:	15.50	CU	GPA 3.76	
Spring 2009		ENGINEERING & APPLIED SCIENCE PHD				
BE	699	BIOENGINEERING SEMINAR	0.50	CU	S	
BE	999	THESIS/DISSERTATION RSCH	3.00	CU	A+	
		Term Statistics:	3.50	CU	GPA 4.00	
		Cumulative:	19.00	CU	GPA 3.81	
Fall 2009		ENGINEERING & APPLIED SCIENCE PHD				
BE	999	THESIS/DISSERTATION RSCH	3.00	CU	A+	
		Term Statistics:	3.00	CU	GPA 4.00	
		Cumulative:	22.00	CU	GPA 3.84	
Spring 2010		ENGINEERING & APPLIED SCIENCE PHD				
BE	999	THESIS/DISSERTATION RSCH	3.00	CU	A+	
		Term Statistics:	3.00	CU	GPA 4.00	
		Cumulative:	25.00	CU	GPA 3.86	
Fall 2010		ENGINEERING & APPLIED SCIENCE PHD				
BE	999	THESIS/DISSERTATION RSCH (2.00)	CU	NR		
BMB	585	WISTAR INST CANCER BIOL	1.00	CU	B+	
		Term Statistics:	1.00	CU	GPA 3.30	
		Cumulative:	26.00	CU	GPA 3.83	
Spring 2011		ENGINEERING & APPLIED SCIENCE PHD				
BE	995	DISSERTATION				
		Term Statistics:	0.00	CU		
		Cumulative:	26.00	CU	GPA 3.83	